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Complete nucleotide sequence of Nootka lupine vein-clearing virus

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Abstract The complete genome sequence of Nootka lupine vein-clearing virus (NLVCV) was determined to be 4,172 nucleotides in length containing four open reading frames (ORFs) with a similar genetic organization of virus species in the genus *Carmovirus*, family *Tombusviridae*. The order and gene product size, starting from the 5′-proximal ORF consisted of: (1) polymerase/replicase gene, ORF1 (p27) and ORF1RT (readthrough) (p87), (2) movement proteins ORF2 (p7) and ORF3 (p9), and, (3) the 3′-proximal coat protein ORF4, (p37). The genomic 5′- and 3′-proximal termini contained a short (59 nt) and a relatively longer 405 nt untranslated region, respectively. The longer replicase gene product contained the GDD motif common to RNA-dependent RNA polymerases. Phylogenetically, NLVCV formed a subgroup with the following four carmoviruses when separately comparing the amino acids of the coat protein or replicase protein: Angelonia flower break virus (AnFBV), *Carnation mottle virus* (CarMV), *Pelargonium flower break virus* (PFBV), and *Saguaro cactus virus* (SgCV). Whole genome nucleotide analysis (percent identities) among the carmoviruses with NLVCV suggested a similar pattern. The species demarcation criteria in the genus *Carmovirus* for the amino acid sequence identity of the polymerase (<52%) and coat (<41%) protein genes restricted NLVCV as a distinct species, and instead, placed it as a tentative strain of CarMV, PFBV, or SgCV when both the polymerase and CP were used as the determining factors. In contrast, the species criteria that included different host ranges with no overlap and lack of serology relatedness between NLVCV and the carmoviruses, suggested that NLVCV was a distinct species. The relatively low cutoff percentages allowed for the polymerase and CP genes to dictate the inclusion/exclusion of a distinct carmovirus species should be reevaluated. Therefore, at this time we have concluded that NLVCV should be classified as a tentative new species in the genus *Carmovirus*, family *Tombusviridae*.

Keywords *Carmovirus* · Genome · *Lupinus nootkatensis* · RNA · *Tombusviridae* · Wild plant virus

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Introduction

New plant viruses continue to be discovered in wild plants [1]. Historically, knowledge of biological and physicochemical properties of a virus was required for placement into a discrete taxonomical category. However, with the advent of molecular biology, genomic sequence data is now most appropriate for definitive identification as a distinct species into classified families and/or genera [2].

The discovery and the characterization of a new virus infecting a wild lupine, *Lupinus nootkatensis* Donn, in the

Talkeetna Mountains of south central Alaska was reported in 2004 [3]. Natural and experimental infections of the virus produced prominent leaf vein clearing and mosaic in leaves of older plants and in seedlings, respectively. Purified virions were described as spherical particles ~30 nm in diameter with a ssRNA about 4.0–4.2 kb. The capsid protein (CP) was estimated to be ~37–40 kDa on SDS-PAGE and western blot analysis, and the double-stranded (ds)RNA profile consisted of three major bands about 4.2, 1.9, and 1.5 kb. Using primers specific to the carmovirus RNA-dependent RNA polymerase (RdRP) [4], a small RT-PCR product was amplified from the viral genome and sequenced. Homology searches in BLAST programs [5] suggested that the virus could indeed be a new carmovirus. The virus was named after its natural host and symptoms, Nootka lupine vein clearing virus (NLVCV), and based on its biological and physicochemical properties, and limited sequence data, was tentatively classified in the genus *Carmovirus*. However, the observed homology between NLVCV and previously published carmovirus was not sufficient for definitive identification since only 503 nt were analyzed, and not the entire genome. Consequently, more sequence data is required for precise taxonomic classification of NLVCV [2].

The genus *Carmovirus* is named after the type species, *Carnation mottle virus* (CarMV), and all members have small isometric nucleocapsids between 32 and 35 nm in diameter. The complete genome sequences of 13 distinct carmoviruses are currently available from gene databanks. The genomic RNAs are monopartite single stranded molecule ranging from 3,803 to 4,266 nt and usually an uncapped short 5′-untranslated region (UTR) that is notably absent in *Turnip crinkle virus* (TCV). In contrast, the 3′-UTR is relatively longer with 223–287 nt that lacks a terminal tRNA-like structure and is not polyadenylated [2]. However, as observed in most virus members in the family *Tombusviridae*, the 3′ terminal sequence can fold into a stem loop structure that has been shown to be required for efficient in vivo replication of the viral RNA [6–11].

The genomic RNA of carmoviruses generally includes four to five open reading frames (ORFs). In CarMV, the first ORF (ORF1) encodes a 28 kDa protein. This protein terminates with an amber codon which may be read-through (ORF1RT) to generate an 86 kDa protein. Both proteins (p28 and p86) are expressed and translated in vivo from the genomic RNA to produce the viral replicase. The next two ORFs (ORF 2 and 3) encode small polypeptides of 7 and 9 kDa, respectively that are expressed from the same subgenomic RNA of 1.7 kb in size. Their function is implicated in virus cell-to-cell and systemic movement in plants. The translation of ORF2 occurs by conventional ribosome scanning while a leaky ribosome scanning mechanism is proposed for the expression of ORF3. The 3′ proximal ORF4 encodes a 38 kDa coat protein (CP) that is translated from its own sgRNA of 1.5 kb in length [12]. The CP has multiple functions and is implicated in genome protection, viral movement [2] and at least for TCV, suppression of gene silencing [13]. Mutagenesis studies in TCV have also shown that all these proteins are essential to retain infectivity [2, 14]. Additional ORFs has been identified in other carmoviruses but their functions remain speculative [15–18].

In this article, we report the complete nucleotide sequence and genomic organization of Nootka lupine vein-clearing virus (NLVCV) [3]. The arrangement and sequence homology of NLVCV's ORFs, and its phylogenetic analysis confirmed that NLVCV belongs to the genus *Carmovirus*, and is most closely related to a subgroup of carmoviruses comprising Angelonia flower break virus (AnFBV) [19], CarMV [12], *Pelargonium flower break virus*, (PFBV) [17], and *Saguaro cactus virus* (SgCV) [20].

Materials and methods

Virus amplification, purification, and RNA extraction

Virions were extracted and purified directly from leaves of naturally NLVC-infected *L. nootkatensis* plants as described by Robertson [3], and stored at 4°C until utilization for electron microscopy or viral RNA extraction. Virus particles were placed onto formvar/carbon-coated copper grid and stained with 1% methylamine tungstate and analyzed with a Jeol transmission electron microscope. Viral RNA was isolated from the virus solution by phenol/chloroform extraction and ethanol precipitation, and stored at –20°C until further utilization.

cDNA synthesis, cloning, and sequencing

The sequence data obtained from the short RT-PCR segment (GenBank accession number, AY584590, 1–503 nt) within the putative polymerase gene of NLVCV [3], was used to design the oligonucleotide NLVCV-1 (5′-TACGGTCGAGAGCTCG-3′) that allowed the amplification of the sequence upstream of this region. The RNA was treated 60 min at 37°C with 2U of tobacco acid pyrophosphatase (Epicentre Biotechnologies) in 50 mM sodium acetate pH 6.0, 1 mM EDTA, 0.1% β-mercaptoethanol, and 0.01% Triton X-100 to remove a possible cap structure at the 5′-extremity. The reactions were stopped with a phenol–chloroform extraction (0.5 vol/0.5 vol), and the RNA was ethanol precipitated. First strand cDNA was then synthesized using the Superscript^{tm} first-strand synthesis system for RT-PCR (Invitrogen) according to the manufacturer's instructions. The cDNA was passed through a column as directed with the QIAquick PCR Purification kit (QIAGEN

Inc.) to eliminate unused primer before proceeding to the addition of a poly-deoxycytidine tail at the 3′-end of the cDNA with terminal transferase (New England Biolabs) as described by the manufacturer. The second strand was synthesized and amplified by PCR using the Expand High Fidelity PCR System kit as described by the manufacturer (Roche Diagnostics) in presence of the NLVCV-1 primer and a poly-deoxyguanosine oligonucleotide. The PCR cycle was programmed as: 2 min at 94°C, followed by 33 cycles of 45 s at 94°C, 45 s at 60°C, and 60 s at 72°C. Amplification products were directly cloned in the vector pCR2.1-TOPO using the TOPO TA cloning kit (Invitrogen). Ligation products were transformed and amplified in DH5α competent cells and the plasmid DNA was extracted with the QIAprep spin miniprep kit (QIAGEN Inc.).

Sequences downstream of the small segment in the polymerase gene previously mentioned were determined by adding a poly-adenine tail at the 3′-end of the genomic RNA using the A-Plus™ Poly(A) Polymerase Tailing Kit as described by the manufacturer (Epicentre Biotechnologies). The first strand synthesis and PCR reaction were performed as described above using a poly-deoxythymidine primer (dT_{24}) and the oligonucleotide NLVCV-3 (5′-CTAGGGTTATCCAGCCGAGG-3′). Cloning was performed as described above for the 5′-portion of the genome. Clones were sequenced with an ABI 3730XL sequencer. The sequenced genome of NLVCV was derived from two clones.

Sequence and phylogenetic analysis

Sequences were screened and compared against the GenBank database at National Center for Biotechnology Information (NCBI) using BLASTn [5]. Viral genomes with contiguous sequences were analyzed and assembled using the Contig Assembly Program (CAP) software [21]. Open reading frames were identified using NCBI ORF finder. Identity/similarity analysis were performed with the program GAP from the Wisconsin (GCG) package version 10.3, using a gap creation penalty of eight and a gap extension penalty of two for amino acid comparisons. The amino acid sequences of the replicase and capsid protein from various carmovirus strains were selected and entered into a multiple alignment generated by the Clustal W software (version 1.83) [22] and corrected through final visual inspection with the SeqLab application (Wisconsin package version 10.3; Accelrys). Phylogenetic analyses were performed for both genes with MEGA version 3.1 [23] using distance methods and the neighbor-joining algorithm. The topological accuracy of the tree was evaluated using 500 bootstrap replicates. Multiple alignments and profile of protein domains were performed with the Pfam database of protein families (version 18), located at the Wellcome Trust Sanger Institute at Cambridge (http://www.sanger.ac.uk/Software/Pfam/) using the default parameters [24]. Whole genomic nucleotide identities (percent) among members of the genus *Carmovirus* and NLVCV were determined with ClustalX [25] with gap opening (10, 10) and gap extension (0.10, 0.20) penalties for pairwise alignment and multiple parameters, respectively. A neighbor-joining tree was constructed with a bootstrap analysis of 1,000 replicates.

Results

Nucleotide sequences and cistrons

The complete genomic RNA of NLVCV has been sequenced and entered in the GenBank databases under the accession number EF207438. The total length of the RNA is 4,172 nt with a GC content of 49%. The genomic organization is similar to other carmoviruses that contains four specific ORFs of the predicted size and arrangement along the genome (Fig. 1A). The first AUG initiation codon is located at nucleotide 60 in a favorable translation context. This ORF encodes a protein 27 kDa (249 aa) that terminates with an amber codon at 807–809 nt. A protein of 87 kDa (774 aa) would be produced with a read-through the termination codon, as suggested for most members of the *Tombusviridae* [2, 26]. Indeed, the sequence surrounding the amber stop codon, AAA-UAG-GGG, is consistent with the consensus sequence AA (A/G)-UAG- G (G/U (G/A)) required for efficient read-through [26]. The highest nucleotide and amino acid (aa) identity detected for

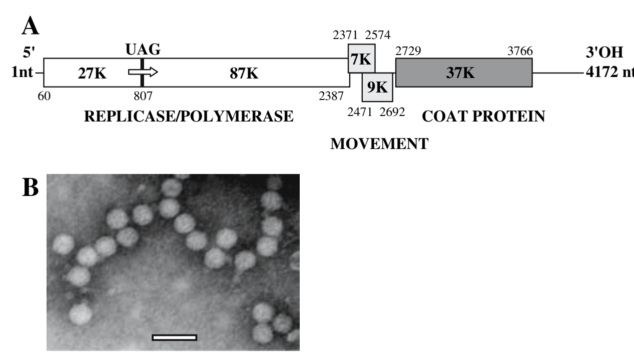

Fig. 1 Particle morphology and genetic organization of Nootka lupine vein-clearing virus (NLVCV). (**A**) The dark line represents the RNA genome starting at nucleotide (nt) 1 on the 5′-terminus and ending at 4,172 nt on the 3′-terminus, with the first and last nt for each of the four open reading frames (ORFs) depicted. The boxes represent the gene product size (kDa) with the functional name directly below. The arrow denotes a readthrough at the amber stop codon (807 nt), forming a larger ORF. (**B**) Electron micrograph of negatively stained NLVCV particles, the bar represents 80 nm

NLVCV p87 coincides with the replicase gene of 13 carmoviruses (37.7–54.2 and 47–61.4%, respectively), and most specifically to CarMV (Table. 1A). Furthermore, the read-through domain of NLVCV p87 contains a GDD motif (1,773–1,781 nt) and conserved surrounding amino acids, which is characteristic of RNA-dependent RNA polymerases [27–29]. Consequently, these observations strongly support the hypothesis that this protein is the RNA-dependent RNA polymerase.

The next two juxtaposed ORF2 and ORF3 encode two small polypeptides of 67 aa (2,371–2,574 nt) and 73 aa (2,471–2,692 nt), for a calculated molecular weight of 7,255 Da (p7) and 8524 Da (p9), respectively. The initiation codon of p7 is located at position 2371–2373, within the 3′-end of the large read-through protein p87. Both p7 and p9 were highly basic proteins, with a pI of 10.07 and 10.76, respectively. The p7 protein showed homology with its respective counterparts found in carmoviruses, all are implicated in virus cell-to-cell movement. Furthermore, amino acids sequence analysis of p7 revealed that this protein contains a RNA-binding domain (GSKG**R**GTG **K**RL**V**AHA**AV**DK) located between its 21 and 39 aa (2,441–2,480 nt), similar to the one identified in *Carnation mottle virus* (CarMV) [30]. The additional presence of the heptanucleotide CAAUUUC (2,565–2,571 nt) upstream of the UGA stop codon of p7 matches the canonical frameshifting motif that is described for other viruses, and specifically with another tentative carmovirus, Pelargonium line pattern virus (PLPV) [8]. A short non-coding intergenic segment (36 nt) was positioned between the p9 and the last protein, CP, near the 3-terminus of the genome.

The CP spanned 1,037 nt (2,729–3,766 nt) with a predicted size of 37 kDa that resembled other carmoviruses. A fairly long non-polyadenylated 3′-non-coding region (406 nt) was in contrast to the relatively shorter 5′-UTR of 59 nt sequences.

Genetic and phylogenetic analysis

The sequenced genome of NLVCV was compared with 13 (three tentative) other distinct virus species in the genus *Carmovirus* with available complete sequenced genomes (Table 1A). When comparing overall percent identities for each gene (nt/aa), the replicase/polymerase represented the overall most conserved gene, ranging from 54.2/61.4 (CaMV) to 37.7/47.5 (*Cowpea mottle virus*, CPMoV), and the CP is the least conserved, ranging from 39.7/50.0 (PFBV) to 24.3/31.7 (*Melon necrotic spot virus*, MNSV). The two small movement genes showed intermediate sequences identity, MP1 (p7) ranged from 47.3/60.0 (PFBV) to 30.8/40.0 (*Japanese iris necrotic ring virus*, JINRV), and the movement protein, MP2 (p9) ranged from 44.1/55.9 (SgCV) to 15.8/21.1 for MNSV. Other genera in the family *Tombusviridae* generally had an overall lower homology with NLVCV when comparing the aa and nt of the replicase and CP (Table 1B). Nucleotide sequence identities (percent) of whole genomes between carmoviruses and NLVCV ranged from 57% (PFBV) to 43% (*Galinsoga mosaic virus*, GaMV) (Table 2).

Phylogenetically, NLVCV formed a well supported subgroup (bootstrap proportions >99%) within the carmoviruses with CarMV, SgCV, AnFBV, and PFBV when separately comparing the aa of the replicase protein (Fig. 2A) and coat protein (Fig. 2B). Analysis of whole genome nucleotide sequences formed the same well supported subgroupings of species (Fig. 3).

Discussion

The genome of NLVCV contains 4172 nucleotides, and thus represents one of the largest viruses in the genus *Carmovirus* when comparing the longest (MNSV, 4,266 nt) and shortest (GaMV, 3,803 nt) genomes. NLVCV shared a similar genetic organization that included gene type, placement and size along the genome (Fig. 1A). Deduced replicative strategies that included two subgenomic RNAs, and conceptual translation of the coding regions for NLVCV resembled those demonstrated for most carmoviruses [2]. NLVCV also shared sufficient sequence identities with the replicase gene, two small movement genes, and the CP genes with carmovirus members to be considered part of that genus (Table 1A). Indeed, phylogenetic analysis of the replicase and CP amino acid sequences revealed that NLVCV formed a distinct subgroup within the carmoviruses that included AnFBV, CaMV, PFBV, and SgCV.

Recent phylogenetic analyses of whole genomes from genera in the family *Tombusviridae* found that carmoviruses did not form an exclusive cluster, but instead clustered, at the most, into subgroups of two species [31]. They found the possibility that extensive gene sharing among some of the genera (i.e., carmoviruses) in the family could occur, and therefore whole genome comparisons gave a more accurate description of relationships. In the carmovirus analysis of whole genome comparisons using nucleotide sequences, NLVCV clustered in one of the three subgroups (Fig. 3). The same species formed subgroups in the phylogenetic analysis of the replicase protein (Fig. 2A) and CP (Fig. 2B).

The physiochemical properties of NLVCV were similar to other carmoviruses, and specifically included a small spherical particle that contained an ssRNA genome about 4.2 kb and a CP ~37–40 kDa [3]. Both of these properties were confirmed from the NLVCV sequence data with a

Table 1 Sequence identity (percent) of the replicase, movement (MP1, MP2), and coat proteins (nucleotide/amino acid) between Nootka lupine vein-clearing virus (Accession number EF207438) and selected members in (A) the genus *Carmovirus** and (B) other genera in the family *Tombusviridae*

Sequence Identity				
Virus, abbreviation (accession number)	Replicase	MP1	MP2	Coat
A				
Carmovirus				
Angelonia flower break virus, AnFBV (NC_007733)	51.0/59.6	32.8/45.9	38.2/48.5	30.8/39.3
Cardamine chlorotic fleck virus, CCFV (NC_001600	42.6/52.5	40.0/47.7	33.8/45.1	29.4/37.7
Carnation mottle virus, CarMV (NC_001265)	**54.2/61.4**	41.0/52.5	34.8/46.4	37.1/47.4
Cowpea mottle virus, CPMoV (NC_003535)	37.7/47.5	35.5/45.2	23.4/32.8	22.6/31.2
Galinsoga mosaic virus, GaMV (NC_001818)	38.3/47.0	30.2/34.9	29.4/35.3	24.8/31.9
Hibiscus chlorotic ringspot virus, HCRSV (NC_003608)	43.6/52.0	35.4/40.0	30.0/45.0	29.8/38.1
Japanese iris necrotic ring virus, JINRV (NC_002187)	44.1/51.5	30.8/40.0	17.6/23.5	30.4/39.2
Melon necrotic spot virus, MNSV (NC_001504)	42.0/51.8	32.2/45.8	15.8/21.1	24.3/31.7
Pea stem necrosis virus, PSNV (NC_004995)	41.7/52.0	37.7/42.6	23.5/23.5	27.0/35.2
Pelargonium flower break virus, PFBV (NC_005286)	51.5/60.7	**47.3/60.0**	29.4/36. 8	**39.7/50.0**
Pelargonium line pattern virus, PLPV (NC_007017)	44.0/51.8	33.3/46.0	38.5/46.2	34.7/45.7
Saguaro cactus virus, SgCV (NC_001780)	50.7/58.4	37.3/49.2	**44.1/55.9**	33.5/44.7
Turnip crinkle virus, TCV (NC_003821)	44.1/52.7	41.3/46.0	31.7/39.7	28.7/37.0
B				
Aureusvirus				
Cucumber leaf spot virus, CLSV (NC_007816)	32.0/41.3			26.3/33.9
Johnsongrass chlorotic stripe mosaic virus, JGCSMV (NC_005287)	36.1/45.7			23.5/32.9
Pothos latent virus, PoLV (NC_000939)	34.0/43.6			23.4/31.1
Avenavirus				
Oat chlorotic stunt virus, OCSV (NC_003633)	32.9/39.6			23.1/29.9
Dianthovirus				
Carnation ringspot virus, CRSV (NC_003530)	29.7/38.3			26.1/36.6
Red clover necrotic mosaic virus, RCNMV (NC_003756)	32.1/39.1			27.0/36.5
Sweet clover necrotic mosaic virus, SCNMV (NC_003806)	32.2/39.5			24.2/34.8
Machlomovirus				
Maize chlorotic mottle virus, MCMV (NC_003627)	37.0/46.1			19.8/27.8
Necrovirus				
Beet black scorch virus, BBSV (NC_004452)	32.7/40.8			21.1/26.3
Leek white stripe virus, LWSV (NC_001822)	33.3/42.2			21.7/27.5
Lisianthus necrosis virus, LNV (NC_007983)	33.9/42.3			22.3/32.0
Olive latent virus 1, OLV-1 (NC_001721)	39.3/47.6			n.a.
Olive mild mosaic virus, OMMV (AY616760)	40.0/48.7			22.8/30.3
Tobacco necrosis virus A, TNV-A (NC_001777)	40.0/49.0			23.1/30.8
Tobacco necrosis virus D, TNV-D (NC_003487)	33.3/42.1			24.8/33.5
Panicovirus				
Panicum mosaic virus, PMV (NC_002598)	36.0/44.1			24.3/34.4
Tombusvirus				
Artichoke mottled crinkle virus, AMCV (NC_001339)	33.3/41.9			22.5/31.7
Carnation Italian ringspot virus, CIRV (NC_003500)	33.8/42.5			23.8/31.7
Cucumber Bulgarian latent virus, CBLV (NC_004725)	33.3/41.7			24.9/33.0
Cucumber necrosis virus, CNV (NC_001469)	34.8/43.4			27.7/37.3
Cymbidium ringspot virus, CymRSV (NC_003532)	34.7/42.3			24.4/33.3

Table 1 continued

Sequence Identity				
Virus, abbreviation (accession number)	Replicase	MP1	MP2	Coat
Maize necrotic streak virus, MNeSV (NC_007729)	34.6/42.8			22.5/29.7
Pear latent virus, PeLV (NC_004723)	33.7/41.8			22.8/32.0
Pelargonium necrotic spot virus, PNSV (NC_005285)	34.0/42.2			22.3/32.3
Tomato bushy stunt virus, TBSV (NC_001554)	33.7/42.7			24.3/34.4

*Highest identities in bold

4,142 nt genome and a computational translation of the CP ORF4 = ~37 kDa.

Three dsRNA species previously extracted from NLVCV infected leaves were about 4.2, 1.9, and 1.5 kb in size (gel analysis) [3]. Although the dsRNAs were not sequenced for definitive identity, the largest dsRNA and two smaller dsRNAs were inferred to represent the whole genome and subgenomic RNAs, respectively. The theoretical predictable sizes of the dsRNAs from NLVCV's sequenced genome are estimated to be 4.2, 1.8, and 1.5 kb [Fig. 1]. However, these values are only speculative, and need to be confirmed by appropriate replication studies. Development of RNA infectious transcripts from NLVCV full-length clones is needed to accurately identify the replication mechanisms and to study gene functions.

Carmoviruses are not serologically related to each other, and therefore an unknown virus can readily be serologically eliminated or included with a specific carmovirus species. In fact, although NLVCV had a relatively high aa sequence identity with the CP of PFBV (50%) and CarMV (47.4%), it was serologically unrelated to either virus [3]. A criteria for inclusion of distinct species in the genus *Carmovirus* is that the aa for the CP and replicase genes are <41 and <52%, respectively [2]. Based on these cutoff percentages for the CP, NLVCV would not be classified as a distinct species, and could be represented as a strain of CarMV (47%), PFBV (50%), PLPV (45.7%), and SgCV (44.7%) (Table 1A). Similarly, when comparing the relatively high replicase (<52%) cutoff point, NLVCV could be a strain of AnFBV (59.6%), CCFV (52.5%), CarMV (61.4%), PFPB (60.7%), SgCV (58.4%), or TCV (52.7%) (Table 1A). It is obvious that the species demarcation criteria for the percent identities of the CP and replicase are too low, and should be reconsidered.

The "natural host range" and "artificial host range reactions" are two other criteria used for species inclusion in the genus *Carmovirus* [2]. NLVCV is confined to one

Table 2 Whole genome nucleotide sequence (percent) identities among members in the genus *Carmovirus* and Nootka lupine vein-clearing virus, NLVCV (see Table 1 for complete virus name and accession number)

	CCFV	TCV	HCRSV	JINRV	AnFBV	PFBV	NLVCV	CarMV	SgCV	PLPV	CPMoV	MNSV	PSNV	GaMV
CCFV	100	69	58	55	48	48	47	47	47	48	47	45	46	43
TCV	69	100	57	54	48	48	47	47	47	47	47	45	46	44
HCRSV	58	57	100	53	48	48	48	46	48	46	48	46	46	45
JINRV	55	54	53	100	47	47	45	46	46	45	47	43	45	42
AnFBV*	48	48	48	47	100	61	56	54	54	51	48	45	45	43
PFBV	48	48	48	47	61	100	57	56	56	52	48	46	46	45
NLVCV	47	47	48	45	56	57	100	53	52	49	46	44	45	43
CarMV	47	47	46	46	54	56	53	100	62	51	47	45	44	44
SgCV	47	47	48	46	54	56	52	62	100	51	46	45	45	43
PLPV*	48	47	46	45	51	52	49	51	51	100	44	43	44	43
CPMoV	47	47	48	47	48	48	46	47	46	44	100	45	45	44
MNSV	45	45	46	43	45	46	44	45	45	43	45	100	63	55
PSNV*	46	46	46	45	45	46	45	44	45	44	45	63	100	55
GaMV	43	44	45	42	43	45	43	44	43	43	44	55	55	100

*Tentative member

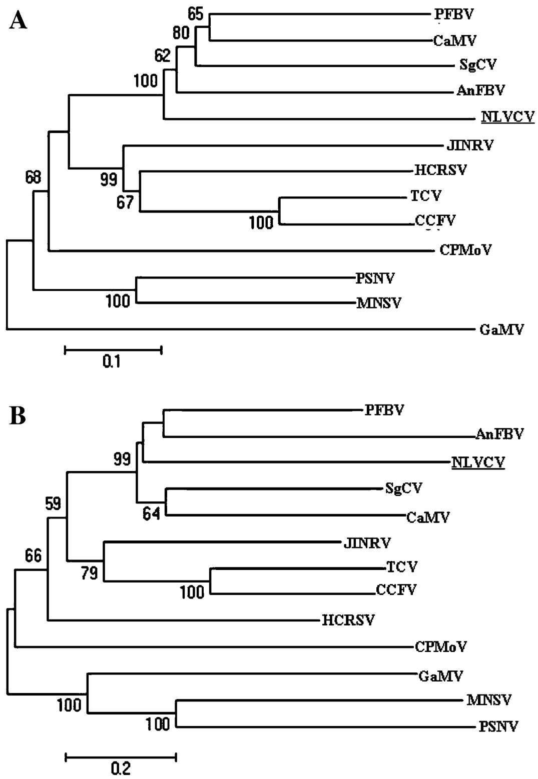

Fig. 2 Phylogenetic analysis of **(A)** the polymerase protein, and **(B)** coat protein from selected members of the genus *Carmovirus* (listed in Table 1), and Nootka lupine vein-clearing virus (NLVCV, accession number EF207438)

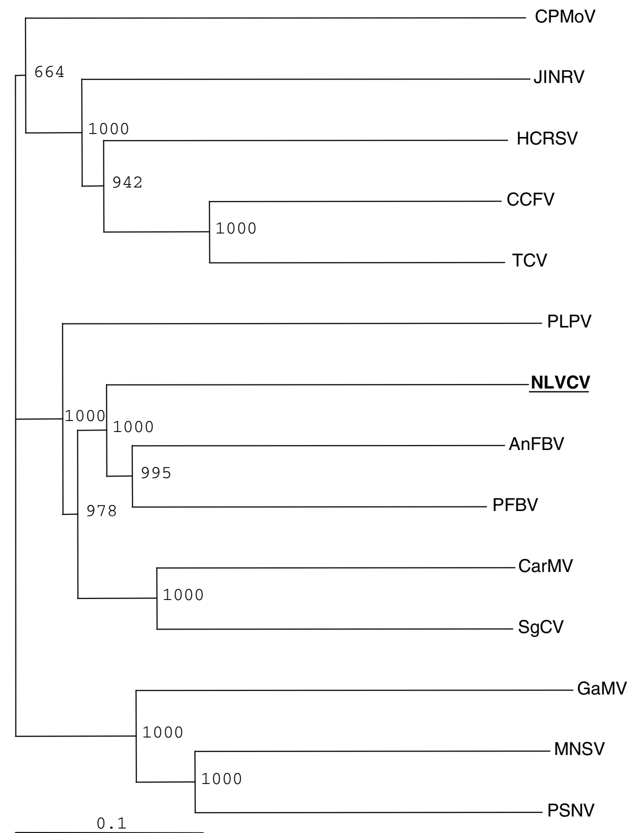

Fig. 3 Phylogenetic analysis of nucleotide sequences of the whole genome from members of the genus *Carmovirus* (listed in Table 1) and Nootka lupine vein-clearing virus, NLVCV, accession number EF207438)

plant species, *Lupinus nootkatensis* L., in nature in a geographically isolated region, and no other virus are known to infect *L. nootkatensis* L. [3]. The experimental host range of NLVCV is limited to species in the family *Fabaceae* [3], and none of the susceptible plant species of NLVCV overlap with plant species that are susceptible to other known carmoviruses [32].

In conclusion, an isolate of NLVCV has been completely sequenced. Based on its genomic organization, sequence identities (percent), phylogenetic analysis, and physiochemical properties, NLVCV is a definitive member of the genus *Carmovirus*, family *Tombusviridae*. The aa identities of the replicase and CP are considered too high for NLVCV to be considered a distinct species when compared with known carmoviruses. However, since NLVCV does not share natural and experimental hosts with other carmoviruses and is serologically unique, we propose that NLVCV be added as a tentative new species in the genus *Carmovirus*, and that the species demarcation criteria be reexamined for the replicase and CP restrictions.

References

1. I. Cooper, R.A.C. Jones, Adv. Virus Res. **67**, 1–47 (2006)
2. S.A. Lommel, G.P. Martelli, L. Rubino, M. Russo, in *Virus Taxonomy: VIIIth Report of the International Committee on Taxonomy of Virus*, ed. by C.M. Fauquet, M.A. Mayo, J. Maniloff, U. Desselberger, L.A. Ball (Academic Press, San Diego, 2005) pp. 907–936
3. N.L. Robertson, Plant Pathol. **53**, 569–576 (2004)
4. S.Y.U. Morozov, E.V. Ryabov, R.M. Leiser, S.K. Kavriev, Virology **207**, 312–315 (1995)
5. S.F. Altschul, T.L. Madden, A.A. Schaffer, J. Zang, Z. Zang, W. Miller, D.J. Lipman, Nucleic Acids Res. **25**, 3389–3402 (1997)
6. D.H. Bringloe, C.W. Pleij, R.H. Coutts, Virology **264**, 76–84 (1999)
7. C.D. Carpenter, A.E. Simon, Nucleic Acids Res. **26**, 2426–2432 (1998)
8. A. Castano, C. Hernandez, Arch Virol. **150**, 949–965 (2005)
9. M.R. Fabian, H. Na, D. Ray, K.A. White, Virology **313**, 567–580 (2003)
10. V. Stupina, A.E. Simon, Virology **238**, 470–477 (1997)
11. R.L. Turner, K.W. Buck, Virology **253**, 115–124 (1999)
12. H. Guilley, J.C. Carrington, E. Balazs, G. Jonard, K. Richards, T.J. Morris, Nucleic Acids Res. **13**, 6663–6677 (1985)
13. F. Qu, T. Ren, T.J. Morris, J. Virology **77**, 511–522 (2003)
14. D.L. Hacker, I.T. Petty, N. Wei, T.J. Morris, Virology **186**, 1–8 (1992)

15. J. Diez, J.F. Marcos, V. Pallas, Arch. Virol. **144**, 1627–1637 (1999)
16. M. Huang, D.C.Y. Koh, L.J. Weng, M.L. Chang, Y.K. Yap, L. Zhang, S.M. Wong, J. Virol. **74**, 3149–3155 (2000)
17. P. Rico, C. Hernandez, Arch. Virol. **149**, 641–651 (2004)
18. X.J. You, J.W. Kim, G.W. Stuart, R.F. Bozarth, J. Gen. Virol. **76**, 2841–2845 (1995)
19. S. Adkins, J. Hammond, A. Gera, C.J. Maroon-Lango, I. Sobolev, A. Harness, M. Zeidan, S. Speigel, Phytopathology **96**, 460–467 (2006)
20. Z. Weng, Z. Xiong, J. Gen. Virol. **78**, 525–534 (1997)
21. X. Huang, Genomics **14**, 18–25 (1992)
22. J.D. Thompson, D.G. Higgins, T. Gibson, J. Nucleic Acids Res. **22**, 4673–4680 (1994)
23. S. Kumar, K. Tamura, M. Nei, Brief. Bioinform. **5**, 150–163 (2004)
24. A. Bateman, L. Coin, R. Durbin, R.D. Finn, V. Hollich, S. Griffiths-Jones, A. Khanna, M. Marshall, S. Moxon, E.L.L. Sonnhammer, D.J. Studholme, C. Yeats, S.R. Eddy, Nucleic Acids Res. **32**, 138–141 (2004)
25. J.D. Thompson, T.J. Gibson, F. Plewniak, F. Jeanmougin, D.G. Higgins, Nucleic Acids Res. **24**, 4876–4882 (1997)
26. L. Harrell, U. Melcher, J.F. Atkins, Nucleic Acids Res. **30**, 2011–2017 (2002)
27. P. Argos, Nucleic Acids Res. **16**, 9909–9916 (1988)
28. G. Kamer, P. Argos, Nucleic Acids Res. **12**, 7269–7282 (1984)
29. E.V. Koonin, V.V. Dolja, Crit. Rev. Biochem. Mol. Biol. **28**, 375–430 (1993)
30. J.F. Marcos, M. Vilar, E. Perez-Paya, V. Pallas, Virology **255**, 354–365 (1999)
31. G.W. Stuart, P.K. Moffett, R.F. Bozarth, Arch Virol. **151**, 1159–1177 (2006)
32. A.A. Brunt, K. Crabtree, M.J. Dallwitz, A.J. Gibbs, L. Watson, E.J. Zurcher (eds.), (1996 onwards). Plant Viruses Online: Descriptions and Lists from the VIDE Database. Version: 20th August 1996. URL http://biology.anu.edu.au/Groups/MES/vide/